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Review

Utility of DNA methylation markers for diagnosing cancer

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\textbf{Abstract}

DNA methylation occurs at the CpG residues and serves as a powerful epigenetic mechanism that negatively regulates gene expression. This process is catalyzed by DNA methyltransferases and occurs within “CpG islands” found in the promoter regions of \(>70\% \) of human genes. Given the important role of DNA methylation in regulating gene expression, un-programmed changes in methylation patterns are expected to either silence or activate transcription of tumor suppressor genes (via hypermethylation) or oncogenes (via demethylation), respectively, and by doing so promote a disease state. In light of the fact that a number of different cancers are frequently associated with hypermethylated tumor suppressor genes together with the observation that tumor derived genomic DNAs are present in various body fluids including serum/plasma, urine, sputum and bronchial lavage, methylated DNA has shown tremendous promise to serve as a robust biomarker for detecting cancer. Over the last several years protocols for capturing small amounts of DNA in circulation have been developed. Once captured, DNA methylation may be readily monitored by restriction enzyme digestion or bisulfite conversion followed by amplification of the desired genomic region with the polymerase chain reaction (PCR). New technologies which employ methyl-binding protein or antibodies that bind specifically to methylated-CpG residues have now enabled investigators to interrogate the status of entire “DNA methyome” of diseased tissue in an efficient and cost-effective manner. In this review, we describe the various tumor suppressor genes that are frequently hypermethylated in different cancers and how these and other methylated loci may be employed as clinically useful biomarkers for diagnosing cancer noninvasively using readily available body fluids.

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1. Introduction

In humans DNA methylation occurs in the dinucleotide 5’-CpG-3’. It has been estimated that over 70\% of genes harbor regions called “CpG islands” in which the frequency of CpG is considerably higher than expected. A vast majority of CpG islands are found in the upstream promoter regions of genes but in others they are located downstream of the transcription start point. Methylation at CpG dinucleotide is catalyzed by three major DNA methyltransferases namely DNMT1, DNMT3a and DNMT3b which covalently attach a methyl group to the C5 position of cytosine residues. DNMT1 is responsible for maintaining genomic DNA methylation patterns and employs hemi-methylated-CpG dinucleotides, produced after DNA replication or repair, as substrate and fully methylates them. DNMT3a and 3b methylate previously unmodified CpG residues and hence are known as \textit{de novo} methylases.

Since DNA methylation silences gene transcription such modifications must be carefully orchestrated during the course of development to ascertain that expression of certain sets of genes is spatially and temporally restricted to specific cell-types. DNA methylation patterns are tissue-specific and “frozen” once development is complete. Un-programmed changes in DNA methylation patterns brought about by gain or loss of function in any of the DNA methyltransferases or demethylases are likely to lead to developmental defects in the growing embryo or a disease state in individuals by altered expression of oncogenes and/or tumor suppressor genes. Nutrition also impacts genomic DNA methylation patterns. Since S-adenosylmethionine (SAM) serves as the source of methyl group which is recycled through the folate and cobalamin dependent pathways,\textsuperscript{1} deficiency of dietary folate and vitamin B\textsubscript{12} is expected to culminate in reduced global levels of DNA methylation.

The negative influence of DNA methylation on gene expression is mediated by methyl-CpG binding proteins (MBDs) that by...
recruiting histone modifying and chromatin remodeling enzymes facilitate the conversion of the loose 10 nm chromatin fiber into a more condensed 30 nm solenoid structure. Eight different MBDS have been identified to date which are MeCP2, MBDS 1, 2, 3, 4 along with Kaiso and its two related proteins ZBTB4 and ZBTB38. With the exception of MBDS 3, all MBDS bind to methylated DNA. Although the functions of various MBDS were considered overlapping, recent studies have shown that MeCP2 and MBDS preferentially target specific gene promoters in prostate cancer derived PC3 cells suggesting that their functions may be mutually exclusive.2 Interestingly, depletion of DNMT1 or DNMT3b has been also found to culminate in preferential loss of DNA methylation at certain genomic loci.3,4

DNA methylation patterns are dynamic during embryogenesis. Around the eight cell embryonic stage, genomic methylation patterns are erased but are then re-established during the implantation stage of the embryo; subsequent embryonic development is associated with additional waves of DNA methylation and demethylation.5,6 The biochemical signals responsible for promoting such dramatic fluctuations in DNA methylation patterns, be they local or genomic loci.3,4 culminate in preferential loss of DNA methylation at certain gene promoters in prostate cancer derived PC3 cells suggesting that their functions may be mutually exclusive.2 Interestingly, depletion of DNMT1 or DNMT3b has also been found to culminate in preferential loss of DNA methylation at certain genomic loci.3,4

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2. Role of DNA methylation in disease

Given the important role DNA methylation plays in regulating and restricting the expression of genes to certain cell-types, it is expected that aberrant changes in DNA methylation are likely to promote a disease state. Although infections as well as environmental factors are known to influence DNA methylation patterns, the mechanisms by which they impact the activity or targeting of DNA methyltransferases, MBDS and/or demethylases to different genomic regions remains unclear. There is now strong evidence suggesting that cardiovascular disease, hypertension, stroke, depression and type-2 diabetes originate early in development because of repeated environmental insults that influence the epigenome.9-11 Similarly, neurological disorders such as schizophrenia, bipolar disorder and autism are associated with un-programmed changes in the epigenome.9-11

Aberrant DNA methylation has also been found to play an important role in cancer development and progression. Genome-wide hypomethylation that is restricted largely to gene-poor regions as well as gene-specific hypermethylation of CpG islands are the two commonest forms of epigenomic modifications that are frequently observed in cancer cells.12,13 Hypomethylation of centromeric DNA promotes genomic instability and appears to increase as cancers progress from non-metastatic to metastatic state.14-17 Cancer cell genomes invariably harbor one or more tumor suppressor genes that are hypermethylated. For instance, silencing of Rb by DNA hypermethylation in retinoblastomas was among the first reports which described how a gene may be inactivated epigenetically.18 Subsequently, a number of other tumor suppressor genes such as VHL, p16, BRCA and hMLH1 have also been found to be hypermethylated in cancers.19,20 Table 1 provides a list of all genes whose promoters have been found to be hypermethylated in various cancers.

3. Using methylated DNA in circulation as cancer biomarker

Clinical utility of even a reliable disease-specific biomarker is undermined if the tissue to be screened requires surgical removal. The ideal biomarker therefore is one which is found in readily available biological samples that can be obtained noninvasively. Due to the high cellular turnover, cancer patients carry elevated levels of free DNA (~200 ng/ml) in their blood. Other body fluids such as urine, bronchoalveolar lavage (BAL), mammary aspiration fluids, saliva, sputum and stools contact tumors are also potential sources of cancer cell DNA. These circulatory DNAs from blood or other body fluids can be captured easily and the status of DNA methylation at various gene promoters interrogated by various methods. According to one study 77% of prostate cancer patients showed GSTP1 hypermethylation in the DNA which was derived from urine as compared to a detection rate of 72% in plasma/serum samples.31 Similarly, analysis of genomic DNA purified from the sputum of lung cancer patients found p16 to be most frequently methylated.27,33 Studies using bronchoalveolar lavage from early stage lung cancer patients have revealed frequent DNA methylation at p16, RARβ, DAPK and MGMT gene promoters.34 Ductal lavage fluid and needle aspirates are also useful sources of tumor DNA which could be employed for detecting breast cancers.28,35 Krassenstein et al. have found a number of genes which were abnormally methylated in genomic DNAs obtained from nipple aspirates of breast cancer patients.35 Abnormally methylated DNAs are also found in the saliva from head and neck, and in stool samples from colon and rectal cancer patients.29,30 Similarly, bladder cancer patients excrete urine that contains hypermethylated DNA. In the p16, E-cadherin and p16 CpG islands; on

4. Methods for determining DNA methylation status of genes

Clinically ideal diagnostic tests are those which can be carried out on readily accessible body fluids (e.g., serum, urine, saliva, etc.). Such tests should be sensitive, specific, reproducible, cost-effective, and be of turn-key nature requiring minimal number of steps so that it could be carried out in a high-throughput manner. Body fluids of cancer patients serve an excellent source of tumor derived genomic DNA which can be used for interrogating the DNA methylation status of either a select panel of genes or the entire genome using a number of different methods. However since these samples invariably contain large amounts of background DNA from normal cells it is essential that the employed diagnostic test be sensitive enough to detect the few available copies of methylated DNA present in the sample.

Initially, DNA methylation sensitive and insensitive restriction endonucleases (e.g., HpaII and MspI, respectively) were employed for detecting CpG methylation at specific genomic loci but since this approach requires large amounts of genomic DNA it is not deemed practical for clinical use. Over the past decade, a number of very sensitive as well as reliable methods have been developed which now allow investigators to detect locus-specific DNA methylation from very small amounts of genomic DNA. Among these, methylation-specific PCR (MSP) has been used most commonly.36 In this assay, genomic DNA is treated with sodium bisulfite which deaminates unmodified cytosines (C) to uracil but...
A fluorescent signal that is proportional to the amount of the probe upon excitation. During the extension phase of PCR the probe contains a fluorophore at the 5' end and a quencher on either end or in the middle, no fluorescence is emitted in the intact molecule. The probe is annealed to the target DNA and is subsequently subjected to polymerase chain reaction (PCR) using primer sets that are strategically designed to span a CpG island for methylated DNA. The main limitation of this approach is that it is only applicable to amplify bisulfite converted DNA: one which is harbored within a restriction enzyme site. The use of bisulfite conversion is particularly advantageous when interrogating the entire epigenome in an open-minded way. In this procedure, genomic DNA obtained from diseased tissue or body fluid is sheared and an antibody (or an MBD) with high affinity for methylated cytosines is used. This step converts all epigenetic information into DNA sequence information. The chemically converted DNA is subsequently subjected to polymerase chain reaction (PCR) using primer sets that are strategically designed to anneal to a specific region within a CpG island of interest and capable of differentiating methylated from unmethylated gene promoters. Although MSP is user-friendly and can detect DNA methylation with impressive sensitivity, it is neither quantitative nor can be performed either in a quantitative or semi-quantitative format, and report nucleic acid amplification in real-time without requiring gel electrophoresis. Additionally, these assays are user-friendly and amenable to automation.

Table 1

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Hypermethylated genes</th>
<th>References</th>
</tr>
</thead>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>53–55</td>
</tr>
<tr>
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<tr>
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<td>58</td>
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<tr>
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<td>Prostate</td>
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</tr>
<tr>
<td>Esophageal</td>
<td>p14\textsuperscript{ARF} (cell cycle), p16\textsuperscript{Dox}</td>
<td>61</td>
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<tr>
<td>Stomach</td>
<td>p14\textsuperscript{ARF} (cell cycle), hMLH1 (DNA repair)</td>
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<tr>
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<td>GSTP1 (detoxification), p16\textsuperscript{Dox}</td>
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</tr>
<tr>
<td>Ovarian</td>
<td>BRCA1 (transcription)</td>
<td>65</td>
</tr>
<tr>
<td>Others</td>
<td>Apoptosis (CASP8), Cell cycle (RB), Differentiation (MYOD, PAX6), Metastasis (MASPIN, TIMP3), Signal transduction (APC, PTEN, AR)</td>
<td>66–74</td>
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Table 2

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Amount of DNA Required</th>
<th>Throughput</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic (MspI/HpaII)</td>
<td>Low</td>
<td>Low-Medium</td>
<td>Moderate</td>
</tr>
<tr>
<td>Hypermethylation sensitive PCR</td>
<td>Low</td>
<td>Low-Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Combined bisulphite restriction analysis (COBRA)</td>
<td>Low</td>
<td>Low-Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Methylation</td>
<td>Low</td>
<td>Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>DNA methylation microarrays</td>
<td>Low</td>
<td>Low-Medium</td>
<td>High</td>
</tr>
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</table>

5. Conclusion

Work over the past decade has shown that DNA methylation biomarkers can not only detect cancer at an early stage but may also be useful for monitoring disease progression during or after treatment. Since tumor derived DNAs are found in various body fluids of cancer patients, they can be obtained noninvasively and screened using various methods to probe presence or absence of DNA methylation at specific loci. Although a number of DNA methylation biomarkers have been identified to date none are reliable enough to be clinically useful. However, development of sophisticated new technology platforms and reagents should eventually lead to the identification of discrete sets of DNA methylation markers (i.e., “signatures”) that are unique to different types of cancers. The clinical utility of DNA methylation markers for diagnosing cancers at an early stage therefore looks promising.

Conflict of interest statement
The authors declare that they have no conflict of interest.

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Ethical approval
None declared.

References


(p16[INK4a] and p14[ARF]) and p53 genes are major targets for inactivation. 


